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≪Research Note≫

Expression of Lipid Metabolism-Associated Genes in Male and Female White Feather Chicken

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Differential lipid metabolic requirements of sexually-mature males and females may influence the regulation of lipid metabolism-associated genes and hence the content of adipose tissue. We measured the expression of eight lipid metabolism-associated genes (fatty acid synthase, *FASN*; acylglycerol- 3- phosphate O-acyltransferase 9, *AGPAT9*; peroxisomal proliferator-activated receptor γ, *PPAR*γ; lipoprotein lipase, *LPL*; carnitine palmitoyl transferase 1 A, *CPT1A*; carnitine palmitoyl transferase 1 B, *CPT1B*; acyl-COA dehydrogenase long chain, *ACADL*; monoglyceride lipase, *MGL*) in eight tissues (hypothalamus, HYP; liver; heart; pectoralis major muscle, PM; gastrocnemius muscle, GAS; abdominal fat, AF; clavicular fat, CF; subcutaneous fat, SF) of five male and five female white feather chickens using real time PCR at 217 d (when the females were at peak egg production). There were no difference between sexes, nor were there sex by tissue interactions for *CPT1A* and *MGL*. In both cases expression was greater for liver than the other tissues. When interactions of sex by tissue were significant, the *FASN* mRNA abundance in HYP, liver, and PM was greater for females than males. There was no sexual dimorphism for any tissue for *PPAR*γ. Overall values were greater for adipose depots than HYP and liver with muscles intermediate for *AGPAT9*. *LPL* mRNA abundance in PM and AF was greater for females than males, with the pattern reversed for heart and SF. *CPT1B* mRNA abundance in GAS and CF was greater for females than males, with the relationship reversed for liver. *ACADL* mRNA abundance in HYP, liver, and GAS was greater for females than males, and lower in PM than males. The results demonstrated that expression of lipid metablism–associated genes varies among sexes in mature chickens depending on the gene and the tissue.

Key words: chickens, gene expression, lipid metablism-associated genes, metabolism path way, real time PCR

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Introduction

The triglyceride content of a cell is regulated by the balance between fat deposition (lipogenesis) and hydrolysis (lipolysis). Accumulation of adipocytes through enhanced rates of cellular differentiation, a process known as adipogenesis, also influences the neutral lipid content of a tissue. Lipid-metabolic genes, including those involved in lipogenesis, can influence the rate of these processes and hence the content of tissue fat. Because of high growth rate and large deposition of fat in the abdomen, the chicken has been used as a model organism for understanding lipid metabolism, fattening and growth (Stern, 2005; Lee *et al*., 2008). Because egg production causes major changes in the metabolism of lipids to meet the demands of yolk formation (Richards *et al*., 2003), it is important to understand underlying genetic mechanisms governing lipid metabolism especially at the peak phase of egg production. Birds have the ability to store large quantities of excess energy (in the form of triglycerides) in the liver, adipose tissue and in the yolk of developing oocytes (Hermier, 1997). Lipogenesis takes place primarily in the liver of birds (Leveille *et al*., 1975) and involves a series of linked enzyme catalyzed reactions including glycolysis, the citric acid cycle and fatty acid synthesis. Fatty acid synthase (*FASN)* has synthesis of long-chain fatty acids functions in lipid metablism pathways. In chickens, like in humans (Dorn *et al*., 2010), FASN primarily occurs in the liver (Demeure *et al*., 2009). Fatty acid esterification includes some holding functional genes such as acylglycerolphosphate acyltransferase 9 (*AGPAT9*) (Bitou *et al*., 1999), and peroxisomal proliferator-activated receptor γ (*PPAR*γ) (Wang *et al*., 2008). Among genes involved in lipid uptake, lipoprotein lipase (*LPL*) has an important role in chicken and mice (Merkel *et al*., 1998). Carnitine palmitoyl transferase 1A (*CPT1A*), carnitine palmitoyl transferase 1B (*CPT1B*), and acyl-COA dehydrogenase long chain (*ACADL*) have fatty acid oxidation functions of skeletal muscle in chicken (He *et al*., 1992; Wu *et al*.,

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2012; Lorzadeh *et al*., 2013), and monoglyceride lipase (*MGL*) has a role in lipolysis of adipose tissue in chicken and mice (Tashler *et al*., 2011). Differential metabolic requirements of sexually-mature males and females may influence the regulation of these genes and body composition in the rat (Kushlan *et al*., 1981; Capel and Smallwood, 1983), fish (Shrestha *et al*., 2013), and hens (Moradi *et al*., 2013). Although there are reports on lipid metabolism-associated gene expression of some tissues in chickens (Karpe *et al*., 1998; Meng *et al*., 2005; Lorzadeh *et al*., 2013), data for sexually-mature males and females is scarce. In this study we investigated expression of lipid metabolism-associated genes in different tissues of white feather chicken at the time of peak egg production, a time of considerable lipid activity.

Materials and Methods

Chickens and Husbandry

In 1999 a white feather chicken population of 2,800 females and 800 males was introduced from the SASSO Company in France by the Poultry Institute, Chinese Academy of Agricultural Sciences. Since then it has been maintained as a closed population conserved in National Chicken Genetic Resources (NCGR). It belongs to a white feather broiler, and was bred from White Plymouth Rocks. The plumage is recessive white with light yellow skin and shank colors. Eggshell color is a pale brown, and the population is segregating for early and late feathering. The average BW of males and females at 42 d is approximately 1,015 and 865 g, respectively, and at 84 d is approximately 2,340 and 1,891 g, respectively, and they are consistent with those for the population as reported by Wang *et al*. (2006). The flock of egg production was at 80% when sampled at 217 d. Egg number to 63 w is 175. The white feather chicken has been used as a parent of quality chicken breeding in China.

The procedures for this experiment were approved by the Institutional Animal Care and Use Committee at Chinese Academy of Agricultural Sciences. Management requirements were in accordance with the white feather chicken meat Breeder Management Guidelines. Chicks were reared in sex-intermingled flocks in floor pens with wood shaving for litter. They were weighed, sexed, and moved to separate floor pens with wood shavings litter at 42 d. Chickens were transferred to individual cages at 140 d.

Diets, fed in mash form, were 20% crude protein (CP), 12.12 MJ/kg metabolically energy (ME) , 1.0% Ca, 0.5% P, 0.44% Met, and 0.95% Lys from hatch to 42 d, and 14% CP, 11.5 MJ/kg ME, 1.0% Ca, 0.45% P, 0.34% Met, and 0.70% Lys from 42 d to 140 d. Thereafter, the diet consisted of 15% CP, 11.5 MJ/kg ME, 3.2% Ca, 0.45% P, 0.37% Met, and 0.90% Lys. These diets have remained constant during selection and are free of antibiotics.

In chickens, after hatch a dramatic increase in fatty acid oxidation occurs in the heart, which has been attributed to an increase in L-carnitine levels and a switch from the liver to muscle (Lorzadeh *et al*., 2013). The liver is the major tissue of lipid synthesis, and the lipid is transported to adipose tissue, the major tissue of lipid storage (Demeure *et al*., 2009). The hypothalamus has the capacity to "talk" with adipose tissue to regulation of energy metabolism (Zhang *et al*., 2014). Muscle is an important part of the chicken and relevant part thereafter life. Therefore, we chose these 8 kinds of tissue: liver, hypothalamus (HYP), heart, pectoralis major (PM), gastrocnemius muscle (GAS), abdominal fat (AF); clavicular fat (CF), and subcutaneous fat (SF) to investigate the genes expression.

At 217 d, 5 males and 5 females were randomly selected and their BW (g) recorded at 4 h post feed-withdrawal. After cervical dislocation and decapitation, the HYP was isolated following the description of Xu *et al*. (2011). It was wrapped in aluminum foil, placed in liquid nitrogen and transferred to −80℃ for storage until RNA isolation. The body cavity was opened and a 300 mg aliquot of the liver (left lobe), heart, PM, GAS, AF, CF, and SF was removed, immediately frozen in liquid nitrogen, and stored at −80℃ for subsequent analysis. CF was the fat above the left side of the clavicle, and SF was obtained from the left side of the chicken from the skin of the peritoneum. The PM was removed from the left side of the keel as a longitudinal strip, while the GAS was isolated from the left leg.

RNA Isolation and cDNA Synthesis

Total RNA was isolated from each of the tissues sampled on individual chickens (300 mg of adipose tissue; about 100 mg of all other tissue types) using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The RNA quality was determined by UV absorbance ratio at 260 and 280 nm. Extracted RNA samples were stored at −80℃.

The complementary DNA was synthesized from 20 ng/μL of total RNA by PrimeScript® 125 reverse transcription Master Mix 126 Perfect Real Time (Takara, Dalian, China) according to the manufacturer's protocol. The reaction volume was 20 μL, and contained 2 μL reverse transcription buffer, 2 μL Random primers, 4 μL dNTP at 2.5 mM, 1 μL reverse transcriptase, and 11 μL RNA (20 ng/μL). Reaction conditions for reverse transcription were as follows: 25℃ for 10 min, 37℃ for 120 min, 85℃ for 5 s, and the kept at 4℃. The cDNA sample was then diluted 8-fold with nuclease-free water and stored at −20℃ before proceeding with real time PCR.

Primers

The chicken-specific primers were designed with Primer Express software (Table 1), for lipid metabolism-associated genes including *FASN, PPARγ, AGPAT9, LPL, CPT1A, CPT1B, ACADL,* and *MGL*. Primers were synthesized by Sangon Biotech Co., Ltd (Shanghai, China). Primer amplification efficiency was validated for *β-actin* and all target genes, with efficiencies that ranged from 95 to 105%. Specificities were confirmed by melting curve analyses (60℃) performed on each sample. *β-actin* was chosen as the housekeeping gene across tissues and between sexes.

Real Time PCR

Real time PCR was performed with Fast SYBR Green Master Mix (ABI 4385612, USA), 96-well thin-walled plates (ABI 4346906, USA), and ABI Prism optical seals (ABI

Table 1. **Primers for real time PCR**[1]

Gene symbol	Sequence 5′ → 3′	Amplicon size/ bp	Location	Genbank ID
FASN	F: CCATTGCACCAGCACTACTCA	59	785-805	NM_205155
	R: ACGAGGCTTAGGGTGTGGAA		824-843	
PPARγ	F: CACTGCAGGAACAGAACAAAGAA	67	806-828	NM_001001460
	R: TCCACAGAGCGAAACTGACATC		851-872	
AGPAT9	F: CCCATAGATGCGATCATTTTGA	61	787-808	NM_001031145
	R: CGTGAACTTGGCCAACCAT		829-847	
LPL	F: GACAGCTTGGCACAGTGCAA	62	298-317	NM_205282
	R: CACCCATGGATCACCACAAA		340-359	
CPT1A	F: GCCCTGATGCCTTCATTCAA	60	1807-1826	NM_001012898
	R: ATTTTCCCATGTCTCGGTAGTGA		1844-1866	
CPT1B	F: TGCTGCTTCCAATTCGACG	68	72-90	DQ314726
	R: TGCAGCGCGATCTGAATG		140-157	
ACADL	F: GACATCGGCACTCGGAAGA	60	148-166	NM_001006511
	R: CCTGGTGCTCTCCCTGAAGA		188-207	
MGL	F: GCGGACGAGCGTAGACTCA	55	1597-1615	XM_414365
	R: GGGAATAGCCTGGTTTGCAA		1631-1651	
β-actin	F: GTCCACCGCAAATGCTTCTAA	58	1176-1196	NM_205518.1
	R: TGCGCATTTATGGGTTTTGTT		1233-1253	

[1] F: Forward; R: Reverse.
FASN=fatty acid synthase, *PPARγ*=peroxisomal proliferator-activated receptor γ, *AGPAT9*=acylglycerol- 3-phosphate O-acyltransferase 9, *LPL*=lipoprotein lipase, *CPT1A*=carnitine palmitoyl transferase 1 A, *CPT1B*=carnitine palmitoyl transferase 1 B, *ACADL*=acyl-COA dehydrogenase long chain, *MGL*=monoglyceride lipase.

4311971, USA) using a 7500 FAST system. Reactions were performed in duplicate for each sample in 10 μL volumes, including 5 μL Fast SYBR Green Master Mix, 0.25 μL forward and reverse primers (5 μM stocks), respectively, 3 μL of diluted cDNA, and 1.5 μL of nuclease free water. The real time PCR conditions were 95℃ for 20 s, followed by 40 cycles at 95℃ for 30 s, 60℃ for 30 s, and final steps of 95℃ 15 s, 60℃ 1 min, 95℃ 15 s for melting curve analysis.

Statistical Analysis

The average of the duplicate Ct values were analyzed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001), where beta actin Ct data were used to calculate ΔCt ($Ct_{target}-Ct_{beta\ actin}$) with the mean of liver for males used as the calibrator sample to calculate ΔΔCt ($\Delta Ct_{X\ group}-\Delta Ct_{calibrator}$) within a gene. Relative quantity (RQ) was calculated ($2^{-\Delta\Delta Ct}$).

Data were analyzed using ANOVA and JMP Version 9.2 (SAS Institute, Inc., Cary, NC, USA). The statistical model included the main effects of tissue, sex, and the interaction between them. The statistical model was $y_{ij}=\mu+S_i+T_j+(ST)_{ij}+\epsilon_{ij}$, where y_{ij} was the dependent variable of gene mRNA abundance, S_i, T_j and $(ST)_{ij}$ were the fixed effect of sex, tissues, and the two way interaction, respectively,with ϵ_{ij} as the random error. Tukey's test was used as a post-hoc test for pairwise comparisons and differences considered significant at $P<0.05$. Body weights were compared by one-way ANOVA with sex as the main variable. When tissue by sex interactions was significant comparisons were by the Paired sample T-test using SPSS 16.0 version. All values were shown as means±SEM.

Results

Body Weights

There was sexual dimorphism for BW ($P<0.05$) at 217 d. Means and SEM for BW at 217 d were 3,009±150 g and 2,257±180 g for males and females, respectively.

Lipid Metabolism-associated Factor mRNA Abundance in Different Tissues of Males and Females

The UV absorbance ratio at 260 and 280 nm of total RNA was 1.8−2.0. Of the eight genes studied there were significant sex by tissue interactions for all but *CPT1A* and *MGL*. For these two genes, values were similar for males and females (Table 2). Among tissues, *CPT1A* values for liver were significantly higher than those for the other tissues which did not differ and for *MGL* value for heart was highest (Table 2).

When interactions of sex by tissue were significant (Table 3) the *FASN* mRNA abundance in HYP, liver, and PM was greater ($P<0.05$) for females than males, with no difference between sexes for any of the other tissues. Within males there was consider overlapping among tissues with a clear significance of HYP>PM. In contrast for females there were clear differences with liver>HYP> the remaining tissues.

Although there was no sexual dimorphism for any tissue for *PPARγ*, the pattern for males was greater expression in adipose than the other tissues. Although this general pattern existed for females the overlap was greater than for males. As with *PPARγ*, there was a lack of sexual dimorphism for *AGPAT9*. Overall values were greater for the adipose depots

Table 2. **Least squares means±SEM in mRNA abundance of genes in tissues where there were no sexual dimorphism or sex by tissue interactions**

Tissue	Gene	
	Carnitine palmitoyl transferase 1 A	Monoglyceride lipase
Hypothalamus	0.25[b]	0.35[b]
Liver	2.09[a]	1.28[a]
Heart	0.78[b]	1.38[a]
Ppectoralis major muscle	0.39[b]	0.17[b]
Ggastrocnemius muscle	0.34[b]	0.17[b]
Abdominal fat	0.27[b]	0.07[b]
Clavicular fat	0.28[b]	0.13[b]
Subcutaneous fat	0.45[b]	0.27[b]
SEM	0.12	0.11

[a, b] Different lower case letters within a column indicates a significant difference between tissues ($P<0.05$).

Table 3. **Least squares means±SEM of mRNA abundance of genes where tissue by sex interactions were significant**

	Fatty acid synthase		Peroxisomal proliferator-activated receptor γ		Acylglycerol- 3-phosphate O-acyltransferase 9		Lipoprotein lipase		Carnitine palmitoyl transferase 1 B		Acyl-COA dehydrogenase long chain	
Tissue	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female	Male	Female
Hypothalamus	1.25[a]*	2.90[b]	0.14[c]	0.45[bc]	0.71[c]	0.84[c]	0.05[c]	0.12[d]	5.60[c]	9.63[b]	1.56[cd]*	5.99[b]
Liver	0.76[abc]*	7.72[a]	0.43[c]	0.49[bc]	1.22[bc]	0.64[c]	0.37[c]	0.24[cd]	3.85[c]*	0.06[c]	3.71[bc]*	7.56[b]
Heart	1.01[ab]	0.93[c]	1.39[c]	0.39[c]	1.39[bc]	1.18[bc]	11.69[a]*	6.23[a]	59.50[a]	73.46[a]	5.60[b]	2.49[c]
Pectoralis major muscle	0.04[c]*	0.24[c]	0.32[c]	0.73[bc]	3.30[ab]	1.57[bc]	0.04[c]*	0.30[d]	46.24[ab]	68.30[a]	1.18[cd]*	0.23[c]
Gastrocnemius muscle	0.99[ab]	0.52[c]	4.72[bc]	1.89[bc]	1.60[bc]	1.56[bc]	0.49[c]	0.28[cd]	45.21[ab]*	534.47[b]	9.48[a]*	15.01[a]
Abdominal fat	0.35[bc]	0.89[c]	7.54[ab]	3.63[bc]	4.96[a]	7.23[a]	0.49[c]*	2.45[bc]	23.67[abc]	16.42[b]	0.60[cd]	0.44[c]
Clavicular fat	0.70[abc]	1.12[c]	11.78[a]	18.00[a]	1.91[bc]	3.44[bc]	4.59[b]	3.42[b]	10.10[bc]*	23.04[b]	0.94[d]	1.84[c]
Subcutaneous fat	0.63[abc]	0.56[c]	7.29[ab]	6.29[b]	3.35[ab]	3.81[b]	0.57[c]*	0.19[d]	35.70[abc]	40.65[b]	2.43[cd]	1.80[c]
SEM	0.18	0.30	1.13	1.27	0.51	0.62	0.75	0.49	8.00	26.69	0.65	0.76

[a, b, c, d] Values within a column with different superscripts differ significantly ($P<0.05$).
* Significant difference ($P<0.05$) between columns.

than HYP and liver with muscles intermediate.

LPL mRNA abundance in PM and AF was greater ($P<0.05$) for females than males, with the pattern reversed for heart and SF. Within males the pattern was heart>CF> the other tissues, although for females general pattern was the same differences were not as delineated. *CPT1B* mRNA abundance in GAS and CF was greater ($P<0.05$) for females than males, with the relationship reversed for liver. Within males abundance was greater for heart, PM, GAS than HYP and liver with adipose tissues intermediate. For females the ordering was GAS>heart, PM, SF, CF, AF, HYP>liver.

Expression of *ACADL* mRNA abundance was greater in the GAS than any of the other tissues. Although *ACADL* mRNA abundance in HYP, liver, and GAS was greater ($P<0.05$) for females than males, in PM it was lower in females than males. For other tissues, males and females were similar. Within males values were greatest for GAS and least for AF with overlapping. In contrast for females the differences were clear with GAS>liver, HYP> the other tissues.

Discussion

In this study the mRNA abundance of eight lipid metabolic genes in eight tissues were compared for males and females, a time when the later were at peak production and lipid metabolism should be great, due to yolk production. *FASN* mRNA abundance in HYP, liver, and PM was greater for females than males. *FASN* catalyzes the last step in fatty acid biosynthesis, it is a major determinant of the maximal hepatic capacity to generate fatty acids by *de novo* lipogenesis (Kusakabe *et al*., 2000). This is especially relevant in chickens where the liver is the major site of *de novo* lipogenesis (Demeure *et al*., 2009), along with a sizable contribution from bone (Yalcin *et al*., 2012). Consistent with those findings, we observed at peak lay that *FASN* mRNA abundance in liver was greater for females than males, and with expression in the liver was greater than in the other tissues. While for males, FASN is expressed in HYP, heart as much as in liver. The metabolic requirements of females and males are thus different, and by this age excess fat deposition is essential to support reproduction during peak lay.

PPARγ is a ligand-activated transcription factor that belongs to the steroid receptor superfamily (Tontonoz and Spiegelman, 2008) and is predominantly expressed in adipose tissue where it is a major inducer of differentiation and adipogenesis (Meng *et al*., 2005; Kilroy *et al*., 2012). Thus, it is not surprising that its mRNA was relatively more abundant in adipose than other tissues.

Fatty acid esterification enhances TG synthesis and may be associated with increased content of muscle fat (Ji *et al*., 2012). Lysophosphatidic acid is further acylated at the sn-2 position by *AGPAT* to form phosphatidic acid (Bitou *et al*., 1999). In the next step, the phosphate group is removed by phosphatidate phosphohydrolase to produce diacylglycerol. Accordingly, in females *AGPAT9* mRNA abundance in adipose depots was greater than in the other tissues measured.

Among lipid uptake genes, the mRNA abundance of the *LPL* gene was highly expressed in the hearts of males. Lipoprotein lipase is a rate-limiting enzyme for the hydrolysis of the triacylglycerol (TG) core of circulating TG-rich lipoproteins, chylomicrons, and very-low-density lipoprotein (*VLDL*). *LPL*-catalyzed reaction products, fatty acids, and monoacylglycerol are transported into adipose tissue and skeletal muscle and stored as neutral lipids (Wang and Eckel, 2009). Our results that abundance was greatest in expression of *LPL* in heart were consistent with previous reports (Karpe *et al*., 1998), although our values were lower in the other tissues.

The two main isoforms of *CPT1* are the liver isoform (*CPT1A*) and the muscle isoform (*CPT1B*) (Park *et al*., 1998). *CPT1A* is expressed in most tissues, but not in skeletal muscle, whereas *CPT1B* is expressed in muscles and adipocytes (Cook and Park , 1999; Jackson-Hayes *et al*., 2003). That mRNA abundance of the *CPT1A* gene was more strongly expressed in liver was consistent with previous reports. Our results that *CPT1B* and *ACADL* mRNA abundance was greatest expression in GAS of females than the other sex and tissue combinations suggest that the subsequent increased fatty acid oxidation may contribute to decreasing lipid deposition in the chicken. *CPT1B* and *ACADL* were differentially expressed in GAS and PM. The increased expression in GAS and reduced expression in PM is consistent with an increased food intake in the females and at the same time reduced fatty acid oxidation in PM yielding a net accumulation of fat storage in PM during peak lay. The results presented in this study were consistent with Ka *et al*. (2013).

Within cells monoglycerides are derived from the hydrolysis of glycerophospholipids or TG. Glycerophospholipids may be degraded by phospholipase C generating sn-1, 2-diacylglycerols, which are further hydrolyzed by sn-1-specific diacylglycerols lipase resulting in the formation of 2-monoglyceride (Gao *et al*., 2010). The enzyme is expressed in most cell types and is considered the rate-limiting enzyme in the degradation of monoglycerides (Karlsson *et al*., 2000). Hence, *MGL* mRNA abundance in liver was greater than all the other tissues except for heart.

In conclusion, our results suggested that *FASN, PPARγ, AGPAT9, LPL, CPT1B,* and *ACADL* mRNA abundance was influenced by sex and tissue combinations when hens are at peak lay. Liver *FASN* plays an important role in the synthesis of fatty acid, and GAS of *CPT1B* and *ACADL* are important to fatty oxidation, particularly in laying females.

Acknowledgments

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